FEBS 27022

FEBS Letters 537 (2003) 215–221



# Role of molecular chaperones in inclusion body formation

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Received 15 October 2002; revised 8 January 2003; accepted 9 January 2003

First published online 10 February 2003

Edited by Thomas L. James

**Abstract** **Protein misfolding and aggregation are linked to several degenerative diseases and are responsible for the formation of bacterial inclusion bodies. Roles of molecular chaperones in promoting protein deposition have been speculated but not proven in vivo. We have investigated the involvement of individual chaperones in inclusion body formation by producing the misfolding-prone but partially soluble VP1LAC protein in chaperone null bacterial strains. Unexpectedly, the absence of a functional GroEL significantly reduced aggregation and favoured the incidence of the soluble protein form, from 4 to 35% of the total VP1LAC protein. On the other hand, no regular inclusion bodies were then formed but more abundant small aggregates up to 0.05 μm$^3$. Contrarily, in a DnaK$^-$ background, the amount of inclusion body protein was 2.5-fold higher than in the wild-type strain and the average volume of the inclusion bodies increased from 0.25 to 0.38 μm$^3$. Also in the absence of DnaK, the minor fraction of soluble protein appears as highly proteolytically stable, suggesting an inverse connection between proteolysis and aggregation managed by this chaperone. In summary, GroEL and DnaK appear as major antagonist controllers of inclusion body formation by promoting and preventing, respectively, the aggregation of misfolded polypeptides. GroEL might have, in addition, a key role in driving the protein transit from the soluble to the insoluble cell fraction and also in the opposite direction. Although chaperones ClpB, ClpA, IbpA and IbpB also participate in these processes, the impact of the respective null mutations on bacterial inclusion body formation is much more moderate.**



*Key words:* Protein aggregation; Inclusion body; Chaperone; *GroEL*; *DnaK*; *Escherichia coli*

## 1. Introduction

The folding into a precise three-dimensional structure is a requisite for protein activity. However, under heat shock and other stresses protein folding can be impaired and folding intermediates then tend to associate through exposed hydrophobic patches. Individual polypeptide molecules are thus being trapped into growing oligomeric aggregates lacking the biological activity. These protein aggregates exhibit a different molecular organisation, such as a fibril structure in the amyloid plaques [1] and a particulate organisation in bacterial inclusion bodies [2]. The precise mechanics of the intermolecular interactions driving the aggregation seeding process remain poorly understood.

Molecular chaperones are essential for the correct folding of a significant fraction of cellular proteins under both physiological and stress conditions [3], acting in a complex network as a main defence against protein aggregation. In this cooperative context, some chaperones exhibit a holding activity, preventing polypeptides from aggregation [4–6], while others show a complementary folding role to assist refolding and solubilisation from aggregates [7,8]. Moreover, chaperones also minimise aggregation by mediating the degradation of proteins that cannot be properly folded [9].

The specific contribution of particular chaperones to this multifunctional folding network has been only partially identified. Aggregates formed upon thermal stress in bacteria have represented a useful model for such functional scrutiny. Upon heat shock, DnaK has been observed as a major protector of aggregation but also as a key element for the solubilisation of small aggregates, in close cooperation with ClpB [6,10–12]. On the other hand, small heat-shock proteins IbpA and IbpB protect heat-denatured proteins from irreversible aggregation [13,14], while GroEL is a main folder element in the multichaperone network [15].

Interestingly, it has been observed that GroEL facilitates the recruitment of $PrP^C$ into pre-existing $PrP^{Sc}$ aggregates [16] and it also promotes $PrP^C$ aggregation in the absence of any template [17]. In the context of conformational diseases, this could be indicative of a role of molecular chaperones not only in the prevention and reversion of aggregation but also in the active formation of structured aggregates. In this regard, perinuclear elements called aggresomes, in which cell chaperones participate as well, have been proven to promote active protein deposition in eukaryotic cells [18,19], these functional complexes being observed as a protective mechanism against the cellular toxicity of misfolded proteins [20]. No analogous mechanism for cell-driven protein deposition has been identified in thermally stressed prokaryotic cells. However, a specific type of aggregates, named inclusion bodies (IBs), is formed by an excluding seeding process in specific sites of the cell cytoplasm [21], and shows an important extent of inner molecular organisation [2]. The building of such organised structures could be compatible with the existence of bacterial mechanisms favouring protein deposition. To explore in more detail this possibility and the eventual participation of molecular chaperones we have here analysed the impact of null mutations in main chaperone-encoding genes on the formation of IBs. Interestingly, DnaK and GroEL appear as antagonist elements in the IB-forming process. While the absence of DnaK results in bigger

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0014-5793/03/$22.00 © 2003 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.
doi:10.1016/S0014-5793(03)00126-1

protein deposits, confirming the role of this chaperone in preventing aggregation, the deficiency in GroEL reduces the extent of protein aggregation and prevents IBs from being assembled from numerous small protein aggregation cores that remain free and independent in the cell cytoplasm.

## 2. Materials and methods

### 2.1. Bacterial strains, plasmids, and culture conditions

Mutant *Escherichia coli* strains were derivative from MC4100 (*araD139* Δ(*argF-lac*)*U169 rpsL150 relA1 flbB5301 deoC1 ptsF25 rbsR*). Inactivating mutations in chaperone genes had been introduced by P1 transduction into MC4100 background to generate strains JGT3 (Δ*clpB::kan*), JGT4 (*clpA::kan*), JGT6 (*zjd::Tn10 groES30*), JGT17 (Δ*ibp::kan*), JGT20 (*dnak756 thr::Tn10*), JGT32 (*dnaK756 thr::Tn10* Δ*clpB::kan*) [22] and BB4564 (*groEL140 zjd::Tn10 zje::ΩSpc$^r$*/*Str$^r$*) [6,23]. BB4565 (*groEL44 zjd::Tn10 zje::Kan*) contained another inactivating mutation in the *groEL* gene, rendering the same GroEL$^-$ phenotype as BB4564 [6,23]. All these strains were transformed with plasmid pJVP1LAC, encoding the misfolding-prone VP1LAC hybrid protein (an amino terminal β-galactosidase fusion) under the control of the lambda $p_R$ and $p_L$ lytic promoters and the temperature-sensitive CI857 repressor [24]. Upon thermal induction of gene expression from 28 to 42°C, VP1LAC is produced as IBs in the cytoplasm of *E. coli* [21]. For some experiments, the related plasmid pJVP1 encoding the non-fused foot-and-mouth disease virus VP1 protein was also used [24]. Luria–Bertani (LB) medium [25] plus the required antibiotics were used for bacterial culture. 3 h after induction of *VP1LAC* gene expression, samples were taken to separate the soluble cell fraction and IBs for analysis. For the in vivo solubilisation experiments, protein synthesis was arrested at this time by both cloramphenicol addition (up to 200 μg/ml) and temperature downshift to 28°C. Then, IB protein evolution was analysed in Western blot by using an anti-β-galactosidase rabbit serum. β-Galactosidase activity was determined as described previously [26]. All the experiments were performed at least in triplicate, and a representative one is shown in the figures.

### 2.2. Separation of the soluble and insoluble protein fractions and IB purification

Cells concentrated up to a pre-defined $OD_{550}$ were ice-jacketed and disrupted by sonication in a Braun Labsonic U device for 10 min with a needle titanium probe. Sonication settings were 20 kHz and 50 W acoustic power with 0.5 s cycles of alternate sonication and resting. Samples were then centrifuged for 15 min at 12 000 rpm, the soluble fraction was stored at −80°C until used and the pellet was treated for IB purification by repeated detergent treatment as described in [27]. Soluble cell fraction and IBs were analysed by polyacrylamide gel electrophoresis and protein bands were detected in Western blot by using a polyclonal anti-β-galactosidase serum.

### 2.3. Light scattering and IB image analysis

Light scattering measurements were made in an Aminco SLM 8000 spectrofluorometer on purified IBs. Scattered light (320 nm) was collected at an angle of 90° of the incident light by integrating in the 315–325 nm window. For image analysis, samples of VP1LAC-producing cells 3 h after the temperature up-shift were fixed with 0.1% formaldehyde and stored at 4°C until use. Photographs were taken at ×1000 magnification factor in a Zeiss (Axioplan D-7082) optical microscope and images processed as described in detail in [21] for IB volume determination. More than 100 individual cells were scanned from each strain for statistics.

## 3. Results

### 3.1. Protein aggregation as IBs is enhanced in the absence of DnaK but largely reduced in a GroEL$^-$ background

The mechanics of the in vivo IB construction and disintegration [28] are still unknown. However, since specific chaperones such as DnaK and ClpB have been proven to detach polypeptides in vitro and also in vivo from certain types of aggregates, these cell proteins (and eventually other chaperones) were presumed to also actively participate in IB solubilisation [29]. Therefore, we investigated the construction and deconstruction of IBs in a relevant set of *E. coli* mutant strains (Table 1). Protein aggregation was determined by light scattering of purified IBs upon recombinant expression of the model hybrid gene *VP1LAC*. In addition, the amount of VP1LAC protein in the isolated IBs was determined by Western blot (Fig. 1). In all these strains, the recombinant protein was the main component of the aggregates up to about 90% of the total protein (data not shown). The coincidence between the profiles of total aggregated protein and VP1LAC amounts (Fig. 1C) indicates that the relative presence of the recombinant protein in the aggregates is not significantly different within the mutant strains and also compared to the wild type, and confirms that VP1LAC is a reliable reporter protein for the analysis of IB formation in different genetic backgrounds. On the other hand, the amount of VP1LAC IB protein was significantly higher in the *dnaK* mutant than in the rest of the strains, and in *groES*, *groEL140* and *clpB dnaK* mutant strains clearly lower than that found in the wild type. The amount of IB protein in the *dnaK* and *groEL140* mutants was also determined for a recombinant, non-fused VP1 protein of 23 kDa, rendering the same profile as that found for VP1LAC (data not shown).

Inversely, the soluble VP1LAC protein in the groEL$^-$ background was more than six-fold higher than in the wild-type cells, while in the *dnaK* mutant the soluble form of VP1LAC was hardly detected (Fig. 2A). Minor variations in the VP1LAC fractioning were observed in the rest of the strains.

### 3.2. Inactivation of DnaK minimises proteolysis of VP1LAC

Interestingly, a VP1LAC degradation fragment of 116 kDa commonly observed in recombinant cells (named 'β-gal-like') was not detected in any of the *dnaK* mutants but in all the other strains (Fig. 2B). This suggested that the absence of DnaK would render VP1LAC more proteolytically stable. In GroEL$^-$ cells, in which most of the recombinant protein

Table 1
*E. coli* strains used in this work

| Strain | Genotype | Phenotype | Reference |
|---|---|---|---|
| MC4100 | *araD139* Δ(*argF-lac*)*U169 rpsL150 relA1 flbB5301 deoC1 ptsF25 rbsR* | wt | [22] |
| JGT3 | MC4100 Δ*clpB::kan* | ClpB$^-$ | [22] |
| JGT4 | MC4100 *clpA::kan* | ClpA$^-$ | [22] |
| JGT6 | MC4100 *zjd::Tn10 groES30* | GroES$^-$ | [22] |
| JGT17 | MC4100 Δ*ibp1::kan* | IbpA$^-$, IbpB$^-$ | [22] |
| JGT20 | MC4100 *dnaK756 thr::Tn10* | DnaK$^-$ | [22] |
| JGT32 | MC4100 *dnaK756 thr::Tn10* Δ*clpB::kan* | ClpB$^-$DnaK$^-$ | [22] |
| BB4564 | MC4100 *groEL44 zdj::Tn10 zje::ΩSpc$^r$* | GroEL$^-$ | [6] |
| BB4565 | MC4100 *groEL140 zdj::Tn10 zje::kan* | GroEL$^-$ | [6] |

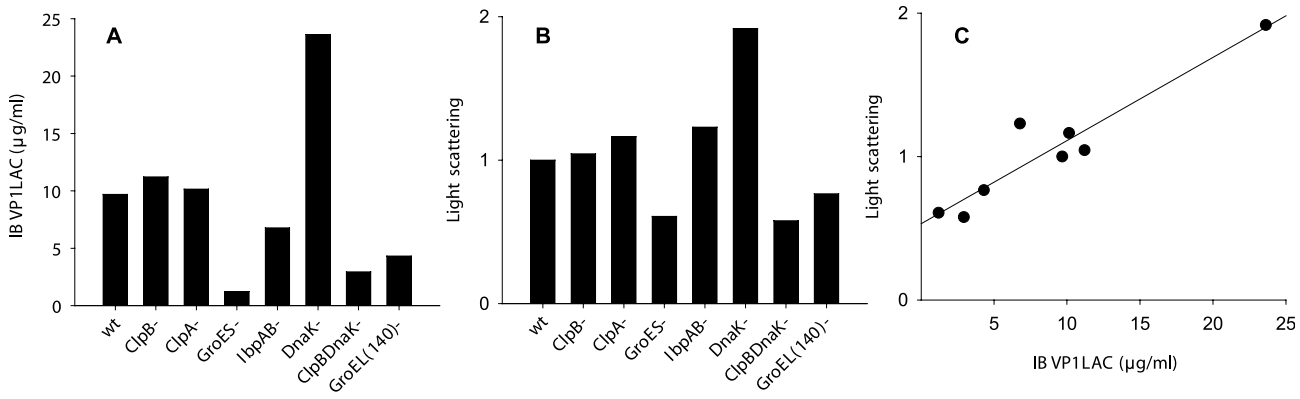


Fig. 1. Amounts of VP1LAC protein in purified IBs 3 h after induction of *VP1LAC* gene expression, as measured by Western blot (A) and light scattering (B) of purified IBs. The growth curves and final biomasses were similar in all the strains in the tested time period. The correlation between both parameters is also shown (C). Statistics are $r = 0.94$; $P < 0.01$.

is soluble, proteolytic stability might be impaired, as observed by the ratio between the truncated and full-length forms of the protein. On the other hand, the divergent impact of *groEL* and *dnaK* mutations on VP1LAC solubility is clearly observed in the protein fractioning as depicted in Fig. 2C.

### 3.3. ClpB *and* DnaK *null mutations have a moderate impact on the in vivo solubilisation of IB protein*

The formation of bacterial IBs is the result of an unbalanced equilibrium between protein aggregation and solubilisation under overproduction conditions [27]. In addition, it has been suggested that chaperones DnaK and ClpB might participate in IB disintegration through the removal of IB proteins [29]. Therefore, the inverse influence of *dnaK* and *groEL* (and also *groES*) null mutations as deduced from the cellular amount of IB protein (Fig. 1) could be due to either a deficient prevention of aggregation and/or a less efficient protein removal from IBs. In particular, the high IB protein amounts found in the *dnaK* mutant strain might be caused by either a favoured protein deposition or by a restricted rate of protein solubilisation in the absence of DnaK. To discriminate between these possibilities, we determined the loss of IB protein upon arresting protein synthesis in cells containing already formed IBs (Table 2). In all the tested genetic backgrounds a lower percentage of protein is removed from IBs than in the wild-type strain (63%), ranging from intermediate (*clpA*, *groES*, *ibpAB*; 42–49%) to high (*groEL*; 32%) negative impact on the VP1LAC release process. In *dnaK* mutants the amount of released protein within a given time period is comparable to that produced by the *groEL140* mutation. This indicates that the higher extent of VP1LAC aggregation observed in *dnaK* compared to the wild type and also to the GroEL$^-$ strains (Fig. 1) is not merely due to a failure in protein solubilisation but rather to altered aggregation pathways that favour protein precipitation in the absence of DnaK. Interestingly, GroEL might have a major solubilising activity on IB protein. This was confirmed by the analysis of IB disintegration also in the GroEL(44)$^-$ background, in which only around 15% of VP1LAC protein was lost from IBs (data not shown), a value still lower than that found in the *groEL140* mutant. Note that, in absolute terms, the amount of protein removed from wild-type IBs does not represent an upper threshold that could eventually restrict protein solubilisation in strains producing bigger IBs.

### 3.4. *The absence of GroEL impairs protein deposition into IBs*

Since DnaK and GroEL chaperones have been proven to be key elements of the cell protein quality control system [30],

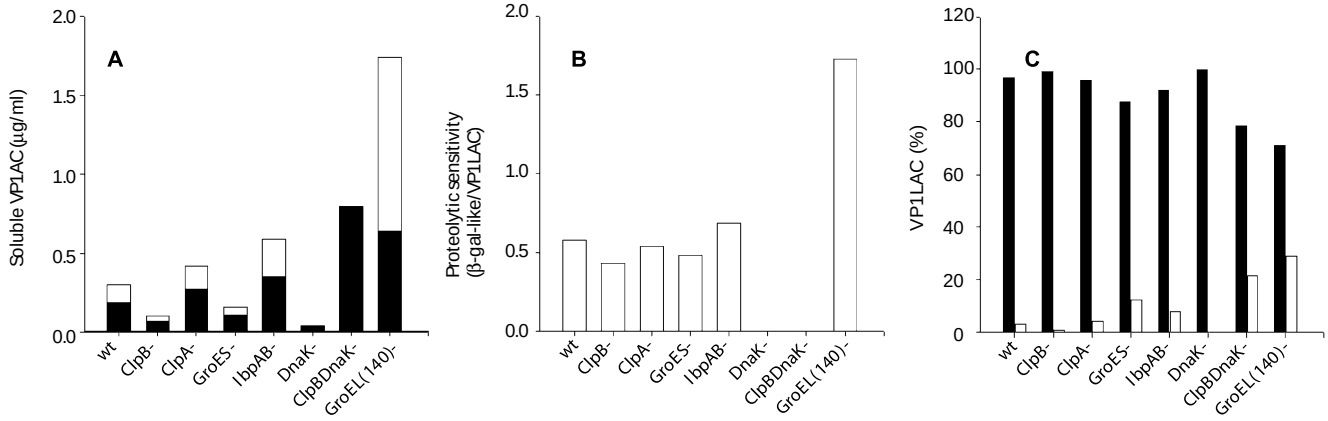


Fig. 2. Amount of soluble VP1LAC protein 3 h post-induction (A). The intact, full-length VP1LAC fusion of 135 kDa is shown as a black bar while a main degradation fragment of 116 kDa (β-galactosidase-like) is shown as a white bar. The quotient between the amount of β-galactosidase-like and intact VP1LAC protein is shown as an index of proteolytic susceptibility (B). The fraction of soluble (white bar) and IB (black bar) VP1LAC protein is also indicated (C). Protein amounts were determined by Western blot of soluble and IB fractions of crude cell extracts.

Table 2
VP1LAC released from IBs in the absence of protein synthesis

| Phenotype | Lost VP1LAC IB[a] (%) | Lost VP1LAC IB[a] (μ/ml) |
|---|---|---|
| wt | 63 | 105.8 |
| ClpB$^-$ | 64 | 124.5 |
| ClpA$^-$ | 42 | 73.9 |
| GroES$^-$ | 49 | 10.34 |
| IbpA IbpB$^-$ | 44 | 51.7 |
| DnaK$^-$ | 34 | 139.4 |
| ClpB$^-$ DnaK$^-$ | 35 | 17.8 |
| GroEL(140)$^-$ | 32 | 23.9 |

[a]Maximal amount of VP1LAC protein lost from IB within a 3 h period after the arrest of protein synthesis. Note that a certain extent of protein re-aggregation can occur under these conditions [27].

their opposite impact on IB formation was analysed in more detail. Protein fractioning was monitored in the *dnaK* and two *groEL* mutants plus in the GroES$^-$ strain. Data at both 3 and 5 h post-induction of *VP1LAC* expression are depicted in Fig. 3. Again, the soluble protein fraction was hardly detectable in the *dnaK* mutant but increasingly abundant in both GroEL$^-$ strains (containing either *groEL140* or *groEL44* inactivating mutations). Interestingly, the absence of GroES does not result in a higher amount of soluble protein as it occurs in the GroEL$^-$ background. The divergent impact of these mutations indicates that the roles of GroEL and GroES in the processing of VP1LAC are not (at least exclusively) cooperative. In agreement with the abundance of soluble protein in GroEL$^-$ cells, the β-galactosidase enzymatic activity in both *groEL* mutants was higher than in the rest of the tested strains (data not shown). This fact proves that, although the absence of GroEL favours the occurrence of soluble VP1LAC, this chaperone is dispensable for its complete refolding to the native conformation.

### 3.5. The regular IB architecture is not reached in GroEL null mutants

The morphological features of IBs formed under the genetic backgrounds depicted in Fig. 3 were determined 3 h after induction of *VP1LAC* gene expression. In wild-type cultures, cells bearing more than two IBs were rarely observed while those containing only one were abundant, representing over 80% of the IB-bearing cells (Fig. 4). This is in agreement with previous observations on the Lon-deficient strain BL21 producing VP1LAC [21]. However, in the *groEL140* mutant, cells containing more than two particles represented more than 60% of the cell population in which aggregation was observed (Fig. 5A), with an average of 5.1 particles per cell. A mere inhibition of cell division as occurring in this strain could not account for the higher number of aggregates since in DnaK$^-$ cells, also showing filamentation, the statistics resemble those of the wild-type strain. In addition, the average volume of IBs formed in DnaK$^-$ is significantly higher than those produced in the wild-type cells (0.38 versus 0.25 μm$^3$), while the aggregates observed in GroEL$^-$ cells only measure 0.05 μm$^3$ (Fig. 5B). We cannot exclude the existence of smaller aggregates in this last strain escaping from the microscopic observation. GroES$^-$ IBs are in the range of 0.18 μm$^3$, close to data obtained from the wild-type cells.

## 4. Discussion

IBs are a particular type of protein aggregates formed in bacterial cells upon expression of recombinant genes at high, non-physiological rates [31]. Structurally, they are porous [32], composed of heterogeneously folded chains [2,33] and organised into a particulate structure [2]. Protein deposition as IBs occurs simultaneously to cell-mediated solubilisation of IB protein [34], the volumetric IB growth being the result of an unbalanced equilibrium of both events that can be redirected towards disintegration upon the arrest of protein synthesis [27]. An important part of the protein removed from IBs undergoes proteolytic attack and is degraded, while a fraction reaches a fully functional form [27]. On the other hand, the formation of IBs and in general protein aggregates is largely enhanced in strains devoid of the protease Lon [24,35]. Altogether, these observations prompt us to observe IBs as an intermediate and transient status of misfolded protein deeply integrated in the cell quality control, in which proteases and chaperones play interconnected roles. Protein aggregation as IBs would then be the result of the inability of chaperones and proteases to process high amounts of misfolded protein, which remains clustered until it can be released to re-enter the quality control. However, the specific cellular proteins involved in the specific processing of IB polypeptides remained unidentified and the specific features of the IB structure allowing the dynamic protein transfer from and to IBs are still to be elucidated.

Recent research on protein aggregation has revealed that a bi-chaperone system consisting of DnaK and ClpB has the capacity to solubilise heat-aggregated proteins (reviewed in [29]). The participation of these and other relevant chaperones in IB formation has been analysed in strains containing null

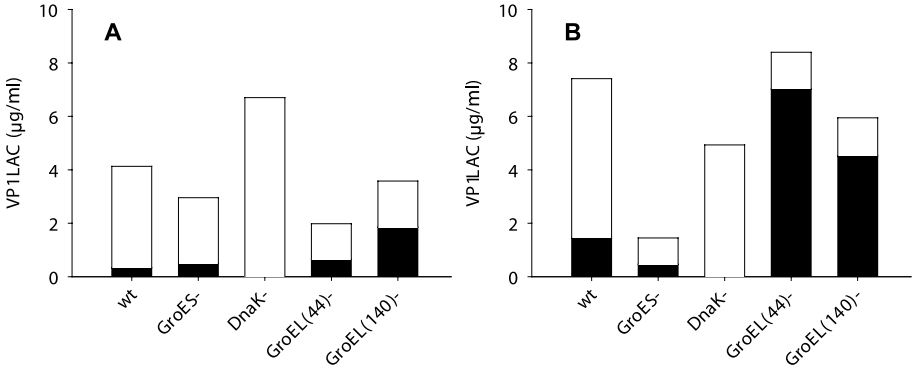


Fig. 3. Partitioning of VP1LAC protein at 3 h (panel A) and 5 h (panel B) after induction of gene expression. Protein was quantified by Western blot for the soluble (black bar) and IB (white bar) fractions of crude cell extracts.

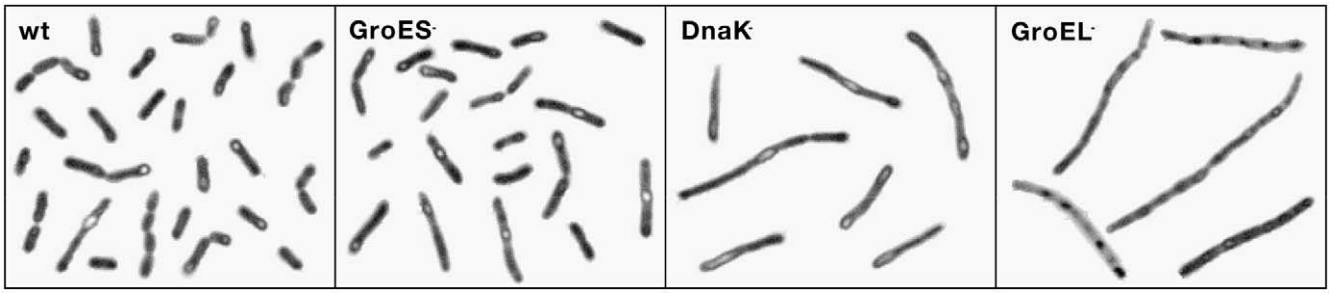


Fig. 4. Micrographs of mutant cells 3 h after the induction of *VP1LAC* gene expression. The GroEL$^-$ cell picture corresponds to strain BB4564 (*groEL140*).

mutations in the encoding genes (Table 1). As was to be expected, the absence of a functional DnaK protein resulted in enhanced amounts of aggregated VP1LAC protein (Figs. 1 and 2) and in large IBs (Figs. 4 and 5). However, this fact is not only depending on impaired solubilisation activities, since the capacity of the DnaK$^-$ strain to remove protein from IB is comparable to that observed in a GroEL$^-$ background (Table 2), in which the deposition of insoluble protein is not particularly favoured (Fig. 1). Interestingly, in the absence of a functional DnaK, the amount of remaining soluble VP1LAC protein is hardly detectable (Fig. 2). Moreover, a commonly observed VP1LAC degradation product, namely the β-galactosidase-like fragment, is absent in both DnaK$^-$ and DnaK$^-$ClpB$^-$ cells but not in ClpB$^-$ (Fig. 2), suggesting that DnaK could be involved in VP1LAC degradation by delivering folding intermediates to proteases. In this context, the DnaK co-chaperone DnaJ has been proven to be involved in the selective breakdown of misfolded proteins by promoting the formation of protease–substrate complexes [9,36]. Also, DnaK itself is necessary for the degradation of particular misfolded proteins and involved in the general proteolytic processing in *E. coli* [3,37].

These results indicate that prevention of aggregation and the favouring of degradation (rather than the disaggregating properties) are the main activities of DnaK involved in IB formation. In this context, the absence of ClpB, participating in the bi-chaperone network for reversion of heat denaturation [6,11], affects neither the total amount of IB protein (Fig. 1) nor the efficiency of protein release from IBs (Table 2). It is not obvious why IB formation is impaired in the double DnaK$^-$ClpB$^-$ mutant (Fig. 1) and why DnaK and ClpB do not cooperate for the dissolution of IBs, while such coordinated activity has been proven on other types of aggregates different from IBs. The low disaggregating efficiency of DnaK-ClpB on big-sized aggregates [38] could account for IBs being recalcitrant to disintegration mediated by these chaperones. On the other hand, the molecular organisation of polypeptide chains in heat- or chemically-denatured protein does not need to be comparable to that of IB protein, where cell chaperones IbpA and IbpB and others are also present and associated with aggregated polypeptides. Therefore, the refolding mechanics and requirements for solubilisation of different aggregates could be dissimilar. In this context, several chaperone-inactivating mutations have observable impairing effects on the release of IB protein (Table 2), proving that this process is the result of cooperative or complementing activities of the multichaperone network.

Unexpectedly, in a GroEL-deficient background, IB formation is largely impaired (Fig. 1), the VP1LAC protein remaining soluble in the cell cytoplasm (Figs. 2 and 3) and retaining enzymatic activity (data not shown). On the other hand, in the absence of this chaperone, regular IBs are not formed (Fig. 4) despite the fact that the total VP1LAC amounts are comparable to those found in wild-type cells (Fig. 3). Contrarily, small and abundant protein aggregates are observed in the cell cytoplasm (Figs. 4 and 5), indicative of a failure in the seeding process in the IB construction. This role of GroEL in promoting protein aggregation and the organisation of small protein aggregates in higher organised structures such as IBs would be irrespective of its well-described folding activities for

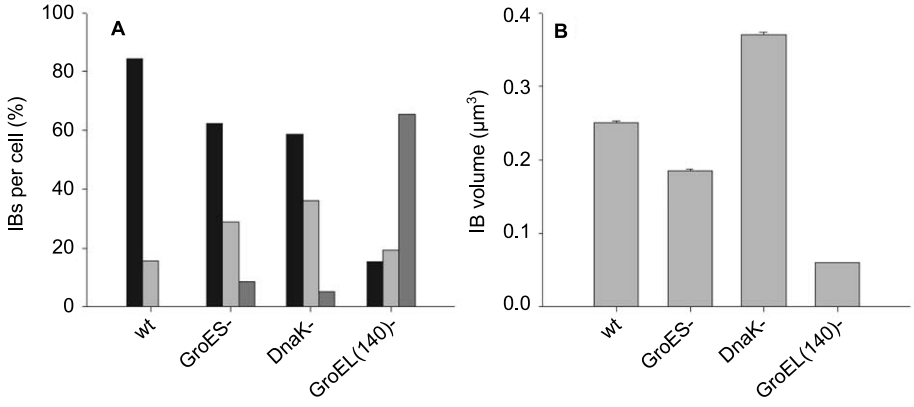


Fig. 5. Number of refractile IB particles per cell, namely one (black bar), two (light grey bar) and more than two (dark grey bar) (A). The average number of aggregates per cell has been calculated for GroES$^-$ (3.0), DnaK$^-$ (3.6) and GroEL(140)$^-$ (5.1). The average IB volume is represented in panel B.

which GroES is required [39]. The divergent impact of GroEL- and GroES-inactivating mutations (Figs. 2–5) indicates a lack of cooperation of these proteins in IB formation. In this context, GroEL without GroES can mediate PrP aggregation in vitro [17]. On the other hand, GroEL is also involved in protein removal from IBs, since the deficiency in this chaperone largely minimises the release of IB protein (Table 2). These results support the idea that GroEL might drive protein transfer not only from the soluble to the insoluble cell fraction but also inversely.

The folding activities of GroEL-GroES are limited by the size of the protein substrate to be encapsulated up to 55 kDa [40], and VP1LAC is a homotetrameric hybrid β-galactosidase enzyme of about 135 kDa per monomer [24]. However, binding of GroEL to proteins larger than 80 kDa for their folding has been reported [41]. In addition, GroEL co-purifies with VP1LAC from crude cell extracts [42] and, in the absence of GroES, it forms in vitro complexes with denatured β-galactosidase [43]. These observations prove not only the dispensability of GroES for GroEL activities leading to IB formation but also the capacity of GroEL to interact with large-sized proteins for processes distinguishable from the encapsulated folding. Although the nature of these interactions still remains unsolved, GroEL association with large-mass substrates would prevent GroES from sealing the GroEL cavity [39]. Moreover, the fact that FMDV VP1 (23 kDa) IBs are also poorly formed in a GroEL$^-$ context (data not shown), indicates that the involvement of GroEL in these IB managing activities might be independent of the molecular mass of the protein. On the other hand, the inefficient VP1LAC solubilisation from IBs in the GroEL$^-$ background (Table 2) suggests that this protein might mediate the transit of misfolded protein from the soluble to the insoluble but also from the insoluble to the soluble states. This would be in agreement with previous experiments performed in vitro and revealing an unusual behaviour of GroEL, in which this chaperone prevented the aggregation of denatured β-galactosidase, but in the presence of ATP, it instead stimulated its deposition into aggregates [43].

In summary, the construction of bacterial IBs is the result of a multichaperone network acting on both aggregation and disaggregation pathways, in which DnaK and GroEL play critical but antagonistic roles. While DnaK prevents IB formation by reducing aggregation and promoting proteolysis of misfolded proteins, GroEL operates protein transit between soluble and insoluble cell fractions and positively participates in IB formation. The antagonism of DnaK and GroEL activities is amazingly exemplified by the volume reduction in IBs formed by the human growth hormone, the increase of the IB number per cell and also the higher amounts of soluble protein found upon over-expression of *dnaK* gene along with the recombinant gene [44], this being a phenotype similar to that reported here for GroEL-deficient mutants. A natural GroEL IB-seeding activity would be comparable to that driving the formation of PrP aggregates in vitro and as suggested to occur in vivo in eukaryotic cells by homologous chaperones during the course of conformational diseases [16,17]. Therefore, GroEL (and eventually the generic Hsp60 chaperone family), in close combination with, or supervised by other cell elements, would act as a key instrument to minimise the toxicity associated with the exposition of hydrophobic domains on misfolded proteins. In bacteria, this protective task might be at least partially accomplished by IB formation.

*Acknowledgements:* We are grateful to Prof. F. Baneyx and Prof. B. Bukau for generously providing mutant *E. coli* strains and to Dr. U. Rinas for helpful comments. This work has been supported by Grants BIO2001-2443 (CICYT, Spain), 2002SGR-00099 (AGAUR, Spain), and by the Maria Francesca de Roviralta Foundation. Part of this paper was written at the GBF (Germany) under a grant from the Deutsche Forschungsgemeinschaft (SFB 578 'Vom Gen zum Produkt') to A.V. M.M.C. received a doctoral fellowship from MEC, Spain.

## References

[1] Fink, A.L. (1998) Fold. Des. 3, R9–23.
[2] Carrió, M.M., Cubarsi, R. and Villaverde, A. (2000) FEBS Lett. 471, 7–11.
[3] Tomoyasu, T., Mogk, A., Langen, H., Goloubinoff, P. and Bukau, B. (2001) Mol. Microbiol. 40, 397–413.
[4] Ehrnsperger, M., Graber, S., Gaestel, M. and Buchner, J. (1997) EMBO J. 16, 221–229.
[5] Veinger, L., Diamant, S., Buchner, J. and Goloubinoff, P. (1998) J. Biol. Chem. 273, 11032–11037.
[6] Mogk, A., Tomoyasu, T., Goloubinoff, P., Rudiger, S., Roder, D., Langen, H. and Bukau, B. (1999) EMBO J. 18, 6934–6949.
[7] Parsell, D.A., Kowal, A.S., Singer, M.A. and Lindquist, S. (1994) Nature 372, 475–478.
[8] Ben-Zvi, A.P. and Goloubinoff, P. (2001) J. Struct. Biol. 135, 84–93.
[9] Huang, H.C., Sherman, M.Y., Kandror, O. and Goldberg, A.L. (2001) J. Biol. Chem. 276, 3920–3928.
[10] Zolkiewski, M. (1999) J. Biol. Chem. 274, 28083–28086.
[11] Goloubinoff, P., Mogk, A., Zvi, A.P., Tomoyasu, T. and Bukau, B. (1999) Proc. Natl. Acad. Sci. USA 96, 13732–13737.
[12] Glover, J.R. and Tkach, J.M. (2001) Biochem. Cell Biol. 79, 557–568.
[13] Kitagawa, M., Miyakawa, M., Matsumura, Y. and Tsuchido, T. (2002) Eur. J. Biochem. 269, 2907–2917.
[14] Kuczynska-Wisnik, D., Kedzierska, S., Matuszewska, E., Lund, P., Taylor, A., Lipinska, B. and Laskowska, E. (2002) Microbiology 148, 1757–1765.
[15] Grantcharova, V., Alm, E.J., Baker, D. and Horwich, A.L. (2001) Curr. Opin. Struct. Biol. 11, 70–82.
[16] DebBurman, S.K., Raymond, G.J., Caughey, B. and Lindquist, S. (1997) Proc. Natl. Acad. Sci. USA 94, 13938–13943.
[17] Stöckel, J. and Hartl, F.U. (2001) J. Mol. Biol. 313, 861–872.
[18] Johnston, J.A., Ward, C.L. and Kopito, R.R. (1998) J. Cell Biol. 143, 1883–1898.
[19] Heath, C.M., Windsor, M. and Wileman, T. (2001) J. Cell Biol. 153, 449–455.
[20] Bucciantini, M., Giannoni, E., Chiti, F., Baroni, F., Formigli, L., Zurdo, J., Taddei, N., Ramponi, G., Dobson, C.M. and Stefani, M. (2002) Nature 416, 507–511.
[21] Carrió, M.M., Corchero, J.L. and Villaverde, A. (1998) FEMS Microbiol. Lett. 169, 9–15.
[22] Thomas, J.G. and Baneyx, F. (1998) J. Bacteriol. 180, 5165–5172.
[23] Zeilstra-Ryalls, J., Fayet, O., Baird, L. and Georgopoulos, C. (1993) J. Bacteriol. 175, 1134–1143.
[24] Corchero, J.L., Viaplana, E., Benito, A. and Villaverde, A. (1996) J. Biotechnol. 48, 191–200.
[25] Sambrook, J., Fritsch, E. and Maniatis, T. (1989) in: Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[26] Ferrer-Miralles, N., Feliu, J.X., Vandevuer, S., Müller, A., Cabrera-Crespo, J., Ortmans, I., Hoffmann, F., Cazorla, D., Rinas, U., Prıvost, M. and Villaverde, A. (2001) J. Biol. Chem. 276, 40087–40095.
[27] Carrió, M.M. and Villaverde, A. (2001) FEBS Lett. 489, 29–33.
[28] Carrió, M.M. and Villaverde, A. (2002) J. Biotechnol. 96, 3–12.
[29] Schlieker, C., Bukau, B. and Mogk, A. (2002) J. Biotechnol. 96, 13–21.
[30] Netzer, W.J. and Hartl, F.U. (1998) Trends Biochem. Sci. 23, 68–73.
[31] Marston, F.A. (1986) Biochem. J. 240, 1–12.
[32] Bowden, G.A., Paredes, A.M. and Georgiou, G. (1991) BioTechnology 9, 725–730.

[33] Oberg, K., Chrunyk, B.A., Wetzel, R. and Fink, A.L. (1994) Biochemistry 33, 2628–2634.
[34] Carrió, M.M., Corchero, J.L. and Villaverde, A. (1999) Biochim. Biophys. Acta 1434, 170–176.
[35] Rosen, R., Biran, D., Gur, E., Becher, D., Hecker, M. and Ron, E.Z. (2002) FEMS Microbiol. Lett. 207, 9–12.
[36] Jubete, Y., Maurizi, M.R. and Gottesman, S. (1996) J. Biol. Chem. 271, 30798–30803.
[37] Sherman, M.Yu. and Goldberg, A.L. (1992) EMBO J. 11, 71–77.
[38] Diamant, S., Ben-Zvi, A.P., Bukau, B. and Goloubinoff, P. (2000) J. Biol. Chem. 275, 21107–21113.
[39] Bukau, B., Deuerling, E., Pfund, C. and Craig, E.A. (2000) Cell 101, 119–122.
[40] Ewalt, K.L., Hendrick, J.P., Houry, W.A. and Hartl, F.U. (1997) Cell 90, 491–500.
[41] Chaudhuri, T.K., Farr, G.W., Fenton, W.A., Rospert, S. and Horwich, A.L. (2001) Cell 107, 235–246.
[42] Boels, K., Carrió, M.M., Arís, A., Corchero, J.L. and Villaverde, A. (1999) Biotechnol. Lett. 21, 531–536.
[43] Ayling, A. and Baneyx, F. (1996) Protein Sci. 5, 478–487.
[44] Blum, P., Velligan, M., Lin, N. and Matin, A. (1992) BioTechnology 10, 301–304.